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TGF-β1-mediated *Smad3* enhances PD-1 expression on antigen-specific T cells in cancer

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Abstract

Programmed Death-1 (PD-1) is a co-inhibitory receptor that down-regulates the activity of tumor-infiltrating lymphocytes (TIL) in cancer and of virus-specific T cells in chronic infection. The molecular mechanisms driving high PD-1 expression on TIL have not been fully investigated. We demonstrate that transforming growth factor-β1 (TGF-β1) directly enhances antigen-induced PD-1 expression through *Smad3*-dependent, *Smad2*-independent transcriptional activation in T cells *in vitro* and in TIL *in vivo*. The PD-1^{hi} subset seen in CD8+ TIL is absent in *Smad3*-deficient tumor-specific CD8+ TIL, resulting in enhanced cytokine production by TIL and in draining lymph nodes and of anti-tumor activity. In addition to TGF-β1's previously known effects on T cell function, our findings suggest that TGF-β1 mediates T cell suppression via PD-1 upregulation in the TME. They highlight bidirectional crosstalk between effector TIL and TGF-β-producing cells that upregulates multiple components of the PD-1 signaling pathway to inhibit anti-tumor immunity.

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Keywords

TGF-β1; *Smad3*; programmed death-1 (PD-1); tumor infiltrating lymphocytes (TIL); tumor microenvironment (TME)

Introduction

Programmed death-1 (PD-1; known as *Pdcd-1* by gene name) is a co-inhibitory receptor induced on T cells by antigenic stimulation (1). PD-1 expression on functional memory CD8+ T cells declines upon the resolution of inflammation and the clearance of antigen during acute infections (2). Conversely, PD-1 expression is maintained on exhausted T cells in chronic infections. In cancer, the PD-1 pathway is highly engaged within the TME, with tumor and immune system cells expressing high levels of the PD-1 ligands, PD-L1 (also known as B7-H1) and PD-L2 (also known as B7-DC), and tumor-infiltrating CD4+ and CD8+ T cells expressing high levels of PD-1 (3,4). Blockade of PD-1 has been effective in prolonging patient survival in melanoma, renal-cell carcinoma (RCC), non-small-cell lung cancers (NSCLC), Hodgkin's Lymphoma and many other cancer types (5-8). Similarly, chronic infection with hepatitis C virus (HCV), hepatitis B virus (HBV) or human immunodeficiency virus (HIV) sustains high levels of PD-1 on viral-specific CD8+ T cells (9-11).

Binding of PD-1 on T cells to its ligands, PD-L1 and PD-L2, can inhibit T cell effector function (12). Pathogen- or tumor-driven inflammation can induce PD-L1 and –L2 expression. For example, PD-L1 is highly expressed on many human tumors (4,13) and its expression is highly co-localized with infiltrating CD8+ T cells in human melanoma patients (14). Similarly, patients with chronic liver disease from HCV and HBV infection also show increased levels of PD-L1 on hepatocytes and Kupffer cells in the liver (15). Elevated PD-L1 and –L2 expression may enhance engagement of PD-1 on T cells and pathogen evasion of host immune responses (4,16-19). The levels of PD-1 on TIL subsets in many cancers are much higher than those seen on normally activated or memory T cells in peripheral blood or in corresponding normal tissue (20). This induction of receptor, together with ligand up-regulation, is likely responsible for the profound inhibition of effector anti-tumor T cell activity in the TME. While interferon-γ (IFN-γ), a T cell effector cytokine, is known to enhance PD-L1 expression on tumor cells (13) and some cytokines have been shown previously to affect T cell expression of PD-1 (21,22), the molecular mechanisms that permit expression of PD-1 on human T cells at very high levels have not been fully elucidated. This is critical to our understanding of PD-1 inhibition of T cell control of tumors or chronic viral infections and modulation of that pathway through immunotherapy.

As part of a cytokine screen to identify those that regulate PD-1 induction on T cells, we found that transforming growth factor-β1 (TGF-β1) modified antigen-driven PD-1 induction to the greatest extent. TGF-β1 is a regulatory cytokine that suppresses immune function in cancers and in chronic viral infections (23-26). The Smad transcription factors transduce signals from TGF-β superfamily ligands that regulate cell proliferation, differentiation, and death through activation of receptor serine/threonine kinases. High serum levels of TGF-β

are associated with poor prognosis in cancer (27,28) and TME-derived TGF-β can suppress anti-tumor T cell responses (29,30). Accordingly, the blockade of TGF-β1 signaling on T cells has been effective in restoring T cell effector functions (31,32). The known suppressive mechanisms of TGF-β1 include *Smad2*/3 dependent inhibition of effector cytokine production by CD8+ T cells in cancer (33) and development of CD4+ regulatory T cells (Tregs) that suppress neighboring effector cells through both contact-independent and -dependent mechanisms (34,35).

Here, we report a novel molecular mechanism of immunosuppression in which TGF-β directly enhances antigen-driven PD-1 gene transcription selectively through Smad3, resulting in enhanced surface expression of PD-1 protein. Utilizing mice with T cells conditionally deleted of *Smad2* or *Smad3*, we found that TGF-β1-enhanced PD-1 expression is abrogated in *Smad3*-deficent T cells. In contrast, *Smad2*-deficient T cells expressed PD-1 at levels comparable to wild-type (WT) mice. This suggests that enhanced PD-1 expression on T cells is predominantly regulated by Smad3. The effect of Smad3 was specific to PD-1 since expression of other inhibitory receptors was not decreased by *Smad3* deficiency. Mice with *Smad3* deficient T cells more effectively controlled tumors in association with loss of the subset of antigen-specific TIL displaying the highest levels of PD-1 and increased TIL and draining lymph node (DLN) cytokine production. PD-1 blockade did not provide further anti-tumor activity beyond that produced by T cell-specific *Smad3* knockout, demonstrating that PD-1 induction by the TGF-β1/Smad3 axis is critical in suppressing anti-tumor T cell function. Thus, our findings suggest that TME-derived TGF-β1 directly augments PD-1 expression on TIL, suppressing CD8+ T cells that engage tumor antigens and enhancing tumor immune resistance.

Results

TGF-β1 enhances PD-1 expression on activated human T cells

To assess the effects of cytokines known to alter T cell development, function, and/or proliferation on PD-1 expression, we isolated CD3+ T cells from healthy donor peripheral blood mononuclear cells (PBMC) and activated them with αCD3/αCD28-conjugated beads in the presence of one of 16 cytokines across a range of concentrations (Supplementary Figure S1a, data shown at 500 ng/mL). The cells were labeled with CFSE to monitor cellular proliferation and PD-1 expression was measured (Figure 1a, representative plots). αCD3/αCD28 induces higher PD-1 expression compared to resting CD8+ and CD4+ T cells, confirming TCR and co-stimulation dependent PD-1 expression (Figure 1a, left and middle graphs). While most of the cytokines tested had no effect or only a modest effect on PD-1 expression upon T cell activation, major enhancement of PD-1 expression was observed with TGF-β1 (Figure 1a middle and right graphs and Supplementary Figure S1a). In conjunction with T cell stimulation, interleukin (IL)-2, IL-6, IL-12, and TNFα induced only modest enhancement of αCD3/αCD28-induced PD-1 expression (Supplementary Figure S1a). Because increased TGF-β 1production is a hallmark of most TME, we chose to further explore its role in PD-1 expression. The co-culture of T cells with TGF-β1 further enhanced PD-1 expression on both CD8+ (Figure 1b, left panel; open symbols) and CD4+ (Figure 1b, right panel; open symbols) T cells versus αCD3/αCD28 alone (Figure 1b, closed symbols)

on all generations (Figure 1b). TGF-β1 did not have any effects on cellular proliferation as measured by CFSE dilution (Figure 1b, black versus open bar graph), suggesting that enhanced PD-1 expression is not simply due to altered cellular proliferation.

While human memory T cell populations such as CMV and EBV-specific T cells express intermediate levels of PD-1, naïve T cells do not express PD-1 (36). To test whether TGF-β1-mediated enhancement of PD-1 expression depends on the basal level of PD-1 expression, we isolated naïve T cells (phenotype CCR7+ CD45RA+) and memory T cells (phenotype CCR7+ CD45RA− or CCR7− CD45RA+) from healthy donors. The cells were activated with αCD3/αCD28-conjugated beads with or without TGF-β1. Although TGF-β1 increased PD-1 expression on αCD3/αCD28 stimulated naive and memory CD4+ and CD8+ T cells (Figure 1c, representative plots), the effect was more pronounced on naïve T cells than on memory T cells for both CD4 and CD8 subsets (Figure 1d, dark and light grey bars). In the absence of αCD3/αCD28, TGF-β1 does not affect the basal levels of PD-1 expression on either naïve or memory T cell subsets (data not shown). This suggests that TGF-β1 enhancement of PD-1 expression is dependent on T cell activation. Furthermore, we found that TGF-β1 increased PD-1 surface expression in a concentration-dependent manner (Figure 1e, left). In contrast, TGF-β1 did not affect expression of the T cell activation marker HLA-DR on T cells (Figure 1e, right), demonstrating that the TGF-β1 effect on PD-1 expression does not simply reflect a general effect on T cell activation-induced antigens. Changes in intracellular and surface levels of PD-1 were positively and directly correlated (Supplemental Figure S1b). Finally, enhanced surface expression of PD-1 was preceded by increased transcription of PD-1, shown as kinetic changes of *Pdcd-1* mRNA levels across different time points (Figure 1f).

TGF-β receptor I (TGF-βRI) kinase activity is critical for TGF-β-dependent enhancement of PD-1 expression

Next, we investigated whether blockade of TGF-β1 signaling can abrogate TGF-β-dependent PD-1 enhancement. TGF-β1 binds TGF-βRI and RII and acts through Smad-dependent and -independent mechanisms (37). Upon binding of the high affinity TGF-βRII by TGF-β1, TGF-βRI and RII heterodimerize and TGF-βRI, a serine-threonine kinase, phosphorylates Smad2/3. To address the role of TGF-β1 receptor signaling, the cells were activated in the presence of TGF-β1 with varying concentrations of either an antibody that blocks the activity of TGF-β1 but not TGF-β2 or TGF-β3 (neutralizing antibody, nAb) (Figure 2a) or a TGF-βRI kinase inhibitor (SB431542) (Figure 2b). Both TGF-β1 nAb and SB431542 decreased TGF-β1-dependent PD-1 expression in a dose-dependent manner, although SB431542 was more effective than TGF-β1 nAb. SB431542-mediated TGF-βR signaling inhibition was shown by the diminished phosphorylation levels of *Smad2* (Figure 2c). Analogous to the effects on surface expression, SB431542 blocked the TGF-β1-dependent increase of *Pdcd-1* mRNA levels (Figure 2d). Given the critical role of nuclear factor of activated T cell (NFATc1) during TCR dependent PD-1 induction (38), we tested whether TGF-β1-dependent PD-1 expression requires NFATc1 by treating cells with cyclosporine A (CsA), a calcineurin inhibitor that exerts its immunosuppressive effects by keeping the transcription factor NFATc1 inactive. We found that CsA completely abrogated not only TCR-dependent but also TGF-β1 enhanced PD-1 expression (Supplementary

Figure S1c), suggesting that TGF-β1 requires TCR-induced NFATc1 activity to enhance the PD-1 expression. Based on the critical role of TGF-βR kinase activity, we next assessed downstream molecules in the TGF-βR signaling cascade.

TGF-β1-dependent Smad3 regulates PD-1 promoter activity

Our data suggest that human PD-1 expression is under direct transcriptional control by TGF-β1 so we hypothesized that TGF-β1 directly modulates human PD-1 promoter activity. We identified putative Smad-binding elements (SBEs), one distal to (SBE-D) and the other proximal to (SBE-P) the *Pdcd-1* transcription start site (Figure 3a). To test this hypothesis, Jurkat T cells were transfected with a luciferase vector containing the 1.9kb-long *Pdcd-1* promoter region and luciferase activity was measured after different treatments. αCD3/αCD28 activation induced PD-1 promoter activity (Figure 3b, grey bars) and mutation in the NFATc1 binding site abrogated such induction (Supplementary Figure S1d, e). Because Jurkat T cells express minimal levels of the TGF-β receptors, the T cells were co-transfected with TGF-βRI and RII plasmids (Supplementary Figure S1f and S1g). The addition of TGF-β1 to αCD3/αCD28 enhanced NFATc1-dependent PD-1 promoter activity (Figure 3b WT and Supplementary Figure S1f). The introduction of site-directed mutations in SBEs (shown in bold letters in Figure 3a, named SBE-D and SBE-P) significantly diminished TGF-β1-driven PD-1 promoter activity and introduction of both mutations (SBE-D/P) further decreased the effect (Figure 3b). To further validate our luciferase-based reporter system, we utilized the chromatin immunoprecipitation (ChIP) assay to verify Smad3 binding to the human *Pdcd-1* promoter. While αCD3/αCD28 did not induce Smad3 binding, addition of TGF-β1 significantly enhanced Smad3 binding to the human *Pdcd-1* promoter (Figure 3c, right graph). This binding was specifically due to TGF-β1 receptor signaling as it was abrogated by treatment with the TGF-βRI kinase inhibitor, SB431542 (Figure 3c, right graph). There was no effect of TGF-β1 on binding of Smad3 to the *Gapdh* promoter (Figure 3c, left graph). We also confirmed that NFATc1 binds to the human *Pdcd-1* promoter following αCD3/αCD28 stimulation and found that this binding was in fact enhanced by TGF-β1 (Supplementary Figure S1h).

TGF-βR1 has serine/threonine kinase activity that phosphorylates Smad2 and Smad3 (39). Smad2 and Smad3 bifurcate the signaling pathway by forming heterodimers with Smad4 (considered a co-Smad) (40,41). Thus, we further investigated whether Smad2 or Smad3 is the dominant regulator of PD-1 promoter activity by using siRNA (Supplementary Figure S2a, b). We found that knock-down of *Smad3* expression (but not *Smad2*) abrogated TGF-β1 enhancement of *Pdcd-1* promoter activity (Figure 3d). In addition, we tested whether specific inhibitor of Smad3 (SIS3), that inhibits phosphorylation of Smad3 but not Smad2, affects PD-1 promoter activity similarly to knock-down of *Smad3* (42). SIS3 inhibited TGF-β1-enhancement of *Pdcd-1* promoter activity (Figure 3e, white bars) without significantly altering NFATc1-dependent *Pdcd-1* promoter activity (Figure 3e, grey bars). Thus, our data collectively showed that *Smad3* is a key mediator of TGF-β1 enhanced *Pdcd-1* promoter activity and increased *Pdcd-1* transcription levels.

Smad3-dependent PD-1 enhancement is conserved in human and murine T cells

To investigate the role of Smad3 on PD-1 T cell surface expression, we treated human CD3+ cells with SIS3 and found that SIS3 treatment decreased PD-1 surface expression on both human CD4+ (Figure 4a, left panel) and CD8+ (Figure 4a, right panel) T cells in a dose-dependent manner. Next, we investigated whether Smad3 deficiency can abrogate TGF-β1-dependent PD-1 expression on murine T cells. CD4+ T cells were isolated from WT, *Smad2* f/f; *Cd4*-cre (*Smad2* cKO), and *Smad3* f/f; *Cd4*-Cre (*Smad3* cKO) mice and activated with αCD3/αCD28 with or without TGF-β1 (Figure 4b). Cre-mediated gene knock-out of *Smad2* and *Smad3* in CD4+ T cells was confirmed by western-blot analysis (Supplementary Figure S3a). Consistent with our human T cell findings, TGF-β1 minimally increased PD-1 expression on *Smad3* cKO CD4+ T cells compared to WT CD4+ T cells as shown in both representative histogram (Figure 4b, left) and PD-1 MFI (Figure 4b, right). In contrast, *Smad2* cKO CD4+ T cells maintained high PD-1 expression in response to TGF-β1 (Figure 4b). Similarly, when WT, *Smad2* cKO, and *Smad3* cKO OT-1 (ovalbumin-specific CD8+ T cells) cells were activated with type-I ovalbumin (Ova) in the presence of TGF-β1, *Smad3* cKO OT-1 showed decreased PD-1 expression (Figure 4c). In contrast, the expression of lymphocyte-activation gene3 (LAG3), another inhibitory receptor, decreased on *Smad3* cKO OT-I and OT-II (Ova-specific CD4+ T cells) cells activated in the presence of TGF-β1, suggesting that TGF-β1 has differential effects on inhibitory receptors (Supplementary Figure S3b). Taken together, the *in vitro* results in both human and murine T cells support the notion that TGF-β1 enhancement of PD-1 transcription is dependent selectively on Smad3.

Tumor-infiltrating Smad3 cKO *CD8+ T cells have decreased PD-1 expression*

PD-1 is highly expressed on TIL and its high expression is associated with decreased effector function in advanced stage human cancer (3,43-46). Given that tumor-microenvironment-derived TGF-β1 can suppress anti-tumor immunity (30,31), we hypothesized that Smad3 contributes to high TIL PD-1 expression. In order to investigate whether TGF-β1 regulates PD-1 expression through Smad3 *in vivo*, WT, *Smad2* cKO, and *Smad3* cKO mice were challenged with B16 melanoma and PD-1 expression was assessed on TIL. Smad2 and Smad3 are known suppressors of T cell function (33), and growth of B16 melanoma in *Smad2* and *Smad3* cKO mice was indeed significantly delayed (Figure 4d). Although both *Smad2* cKO and *Smad3* cKO mice had comparably decreased volumes of B16 melanoma versus WT, the PD-1^{hi} subset population was significantly lower on *Smad3* cKO CD8+ TIL, but not on *Smad2* cKO CD8+ (Figure 4e). In contrast, LAG3 expression was significantly enhanced on *Smad2* cKO CD8+ TIL but unaffected by *Smad3* cKO (data not shown). PD-1 expression on CD4+ TIL was not significantly different among WT, *Smad2* and *Smad3* cKO groups although the level of PD-1 expression was much lower on CD4 T cells than on CD8 T cells in all three mouse groups (data not shown). The lack of a significant difference in PD-1 expression on CD4+ T cells *in vivo* could be due to the minimal fraction of antigen-specific CD4+ effector T cells in the tumor microenvironment. Alternatively, because PD-1 is expressed by Tregs (47,48) that constitute the majority of CD4+ TIL in B16 melanoma, most CD4+ TIL may not be specific for tumor antigens. Supporting this notion, we found that the majority of CD4+ PD-1+ TIL express Foxp3 (Supplementary Figure S3c). However, in the presence of TCR signaling *in vitro*, Foxp3+

and Foxp3− CD4+ T cells are capable of enhancing PD-1 expression to the same extent in response to TGF-β1 (Supplementary Figure S3d,) and the FoxP3-CD4+ TIL did not express lower levels of PD-1 in the *Smad3* cKO than in the WT mice (data not shown).

In order to test whether TGF-β1 induced Smad3 activation up-regulated PD-1 on tumor antigen-specific T cells, we utilized B16 melanoma cells stably expressing ovalbumin as a model tumor antigen (B16-Ova). CD45.1 WT mice were challenged with B16-Ova, and CD45.2 OT-1 cells from WT and *Smad3* cKO OT-1 mice were adoptively transferred into the tumor-bearing mice after tumor cells became palpable. The tumor growth was monitored and lymphocytes infiltrating into tumors were harvested after 5 days. We found that transferred *Smad3* cKO OT-1 cells limit tumor growth more effectively than WT OT-1 cells do (Figure 5a). Consistent with our *in vitro* data, neither *Smad3* cKO TIL nor *Smad2* cKO TIL showed significantly increased cellular proliferation by CFSE (Figure 5b, 5c) when gated on CD45.2+ (antigen experienced) donor cells. When a proliferated subset (i.e. CFSE negative subset) is further gated, *Smad3* cKO TIL show significantly fewer of the PD-1^{hi} expressing T cells highly characteristic of WT TIL (Figure 5d, top). This effect of Smad3 was specific to PD-1 since there was no reduction in LAG3^{hi} subset in *Smad3* cKO TIL (Figure 5d, bottom). PD-1^{hi} expressing cells did not decrease in *Smad2* cKO OT-1 (Figure 5e, top), further supporting that Smad3 is a critical mediator of PD-1 expression in the tumor microenvironment. In contrast, *Smad2* cKO TIL maintained high levels of PD-1 (Figure 5e, top), which is consistent with our observation in polyclonal CD8+ T cells (Figure 4e). Similar to TIL, LAG-3 expression on OT-1 cells was not significantly affected in *Smad3* cKO mice. Conversely, PD-1 and LAG3 expression on T cells in draining lymph nodes (DLN) and in non-draining lymph nodes (NDLN) was comparable between WT and *Smad3* cKO OT-I (Supplementary Figure S4a,b) or *Smad2* cKO OT-I (Supplementary Figure S4c,d), suggesting that the effect of TGF-β1 is specific to the tumor-microenvironment. To confirm the specificity to the tumor microenvironment, WT and DNTGFβRII Tg+ mice were challenged with B16 melanoma and tumor volume was measured. In association with enhanced anti-tumor immune responses in DNTGFβRII Tg+ mice (Supplementary Figure S4e), decreased PD-1 expression was also observed on CD8+ TIL, but not on T cells from DLN (Supplementary Figure S4f). As in *Smad2* and *Smad3* cKO mice, the DNTGFβRII Tg + mice did not show significant decreases in LAG-3 expression on TIL or DLN T cells (Supplementary Figure S4g).

TGF-β1/Smad3-dependent enhanced PD-1 expression is associated with decreased T cell function

We next assessed the effect of *Smad2* and *Smad3* cKO on T cell function. The number of TIL obtained in the B16 model is smaller than in some other tumor models and isolation of TIL from the tumors harvested from the *Smad2* and *Smad3* cKO mice particularly challenging. To confirm our findings with B16 melanoma and to permit functional analysis of TIL and T cells from the DLN, we used a cancer model with more abundant TIL, the MC38 colon cancer model. WT, *Smad2* cKO, and *Smad3* cKO mice were challenged with MC38 colon cancer and PD-1 expression assessed. As with the B16 melanoma (Figure 4d), tumor growth in *Smad2* and *Smad3* cKO mice was significantly delayed (Figure 6a). In contrast to CD8+ TIL, the PD^{hi} subset population was absent in CD4+ TIL in all groups and

differences could not be assessed. As in B16 melanoma, the MFI of PD-1 on CD8 + TIL was significantly lower in the *Smad3* cKO than in the WT or *Smad2* cKO mice (Figure 6b). We performed intracellular cytokine staining (ICS) to examine CD8+ T cell production of IFN-γ, TNF-α, IL-2, Foxp3, and granzyme B in WT and *Smad3* cKO mice, but had insufficient TIL to perform ICS analysis for the *Smad2* cKO group. We saw no significant differences between the *Smad3* group and the *WT* group in Foxp3 or granzyme B levels when examining TIL or DLN. However, in the *Smad3* cKO group compared to the *WT* group, there was significantly higher production of (tumor necrosis factor-α (TNF-α) from CD8+ TIL and of IFN-γ and IL-2 in the DLN (Figure 6c). Thus, decreased PD-1 expression secondary to the loss of *Smad3* signaling in TIL and T cells in the DLN is associated with increased production of multiple cytokines and functionality.

TGF-β1/Smad3-dependent enhanced anti-tumor effects involve PD-1 expression

TGF-β1 is known to inhibit CD8+ T cell effector function through Smad2/3 (33) and many different mechanisms (37). Our data provide evidence that enhancement of PD-1 expression represents a newly defined mechanism through which TGF-β1/Smad3 suppresses T cell function. In order to address how significant the impact of Smad3-mediated PD-1 up-regulation is on tumor evasion of T cell responses, we treated *Smad3* cKO mice bearing B16-melanoma with an anti-PD-1 blocking antibody (αPD-1) previously shown to have therapeutic efficacy in WT mice bearing B16-melanoma (49,50). If the effect of *Smad3* cKO on tumor growth is mediated through a mechanism other than PD-1 or if the effects of *Smad3* cKO on PD-1 expression are not sufficient to negate that mechanism of tumor evasion, treatment with αPD-1 would confer additional therapeutic benefits in *Smad3* cKO mice. To assess this, WT and *Smad3* cKO mice were challenged with B16 melanoma cells and were given either isotype-matched IgG or αPD-1. We found that the tumor volume was decreased with αPD-1 compared to IgG treated WT mice (Figure 7, WT) attesting to the general role of the PD-1 pathway in immune resistance in this tumor model. In contrast, αPD-1 had no effect on tumor growth in *Smad3* cKO mice (Figure 7, *Smad3*).

Discussion

We show here that TGF-β1, signaling selectively through *Smad3* transcriptional regulation, significantly up-regulates PD-1 in the context of TCR engagement. We show that this mechanism is important in generating a PD-1^{hi} population of T cells in the tumor microenvironment, where TGF-β1 expression is commonly very high. Thus, up-regulation of both PD-1 ligands and the PD-1 receptor itself contribute to PD-1 pathway mediated tumor immune resistance.

PD-1 expression can be differentially regulated by the environmental context in which a T cell encounters antigen. Upon activation, NFATc1 transiently induces PD-1 expression on T cells (38). Once PD-1 expression is induced, it is sustained in chronic infections or toleragenic environments (2), but high level PD-1 expression is not achieved when antigen is encountered in an inflammatory environment, such as in *Listeria monocytogenes* infection (51). Further supporting the notion that the level of PD-1 expression is context-dependent, there has been emerging evidence that cytokines can regulate NFATc1-induced PD-1

expression. Interferon-α (IFN-α) promotes PD-1 expression on murine T cells through signal transducer and activator of transcription-1 (STAT-1) mediated transcriptional regulation of PD-1 gene expression (21,22). IL-6 also increases PD-1 expression through a STAT3-dependent mechanism in murine CD8+ T cells *in vitro* (52), and we found the similar regulation in human CD4+ and CD8+ T cells (Supplementary Figure S1a). IL-12 has differential effects on PD-1 *in vivo* and *in vitro*. IL-12-conditioned tumor-specific memory CD8+ T cells have lower PD-1 expression *in vivo* with stronger anti-tumor immune responses (21). In contrast, we have found that IL-12 increases PD-1 expression on human CD4+ and CD8+ T cells *in vitro*, consistent with other's findings on murine CD8+ T cells (52). Thus, while our data agree with the literature that IL-6 and IL-12 modulate PD-1 expression, TGF-β1 has the greatest effect on PD-1 expression, which has not been shown previously. The effects of some cytokines could be greater *in vivo* than we observed *in vitro*. However, our *in vivo* data demonstrating that loss of TGF-β1 signaling has a profound impact on high level PD-1 expression upon TCR engagement while signaling from other cytokines remains intact suggest that TGF-β1 signaling through *Smad3* is the major regulator of T cell expression of PD-1 and function.

The data on the effect of other cytokines on PD-1 expression also collectively show that the regulatory mechanisms of PD-1 expression are highly conserved between human and mouse. This is further supported by high sequence homology between human and murine *Pdcd-1* proximal promoter regions including the NFATc1 binding site (52). We demonstrate that Smad3-dependent PD-1 regulation is also conserved in showing that Smad3 has the greatest effects on PD-1 expression on both human and murine T cells. NFATc1 was previously shown to be critical for PD-1 induction in mice (38) and mutation of antigens such that the TCR is no longer engaged results in a decline in human PD-1 expression in chronic infection with HCV or HIV (10,53). Supporting previous findings that PD-1 expression depends on TCR engagement, we find that antigenic stimulation is required for TGF-β1 to enhance PD-1 expression and that NFATc1 binding to the human *Pdcd-1* promoter following αCD3/αCD28 is enhanced by TGF-β1. Furthermore, TGF-β1 enhances PD-1 expression on both CD4+ and CD8+ T cells regardless of their naïve or memory status, although its effect was more pronounced on naïve T cells than on memory T cells.

Our proliferation assays showed that TGF-β1-mediated PD-1 enhancement is independent of cellular proliferation. In contrast to other's findings for which Smads were proposed to play a role in TGF-β1 suppression of T cell proliferation (54), we found that TGF-βRI-dependent signaling, as demonstrated by phosphorylation of Smad2 (Figure 2c), did not result in suppression of T cell proliferation. TGF-β1-mediated suppression of proliferation can be overcome by CD28-mediated co-stimulation, and it is possible that αCD3/αCD28 used in our *in vitro* culture system masked inhibition (55). However, neither *Smad2* cKO OT-1 nor *Smad3* cKO OT-1 showed significantly altered proliferation *in vivo* (Figure 5bc). Nevertheless, we observed that isolated *Smad3* cKO CD4+ T cells have increased IL-2 expression compared to WT litter mates when activated with αCD3/αCD28 (data not shown), consistent with previous reports (56).

Others have suggested a potential association between TGF-β1 signaling and high PD-1 expression, but the direct causal relationship, the molecular mechanism, and biological

implications of this association have not ever been characterized (32,57,58). TGF-β1 signaling consists of Smad and non-Smad dependent pathways and Smad-dependent gene regulation (Smad2 and Smad3) has been well-characterized (59,60). Some genes are preferentially and exclusively regulated by Smad2 or Smad3 as with *Id1* and *Myc* (61,62). On the other hand, Smad2 and Smad3 can redundantly regulate expression of many genes that are under control of TGF-β1 (63). Our luciferase assay and *in vitro* data suggest that PD-1 regulation is predominantly under the control of *Smad3*. Although our *in vitro* data support a minor role for Smad2 in TGF-β1-dependent PD-1 enhancement, our *in vivo* data clearly demonstrated no enhancement of PD-1 expression through Smad2 with *Smad2* cKO mice showing a small increase rather than decrease in PD-1 expression. The *in vivo* data could reflect enhanced Smad3 activity in compensation for Smad2 deletion in T cells.

Our *in vivo* studies mainly focused on CD8+ T cells because the PD-1 expression difference was greater in CD8+ T cells than in CD4+ T cells *in vivo* and the levels of PD-1 on CD4+ T cells much lower than on CD8+ T cells. Supporting this notion, TGF-β1-suppression of anti-tumor immunity *in vivo* appears to be dependent on CD8+ T cells but not on CD4+ T cells in a murine mouse model (31). This discrepancy could be due to cellular intrinsic difference between CD4+ and CD8+ subset of T cells (64). Our observation that stimulated OT-I and OT-II T cells respond similarly to TGF-β1 *in vitro* (Supplementary Figure S3b) but CD4 and CD8 TIL *in vivo* do not may be explained by an absence of antigenic recognition by CD4+ TIL *in vivo* due to CD4+ T cells being primarily Tregs (Supplementary Figure S3c,d). Alternatively, mechanisms of PD-1 regulation unique to CD4+ T cells may exist *in vivo* given that the Foxp3 negative CD4+ T cells in the *Smad3* cKO mice did not express lower levels of PD-1 than WT. (data not shown).

Interestingly, the effect of TGF-β1/Smad3 on PD-1 expression of CD8+ T cells was specifically on TIL, but not on those originating from tumor DLNs. We did not find the percentage of PD-1^{hi} T cells isolated from the tumor-DLNs in WT mice to be significantly different from that of DNTGFβRII Tg+ and *Smad3* cKO mice (Supplementary Figure S4a, b, f). This may be due to the fact that the PD-1^{hi} CD8+ T cell population prominent in the tumor microenvironment was absent in DLNs in WT mice, and suggests that TGF-β1 levels could be much lower outside of the tumor microenvironment. Supporting this notion, others found that TGF-β1 expression is higher in human head and neck squamous cell carcinoma tissue than in adjacent mucosal tissue (65). Nevertheless, it is yet to be determined whether the dominant source of TGF-β1 in the tumor microenvironment is derived from tumor or T cells. In spite of extensive evidence that TGF-β1 suppresses anti-tumor immunity, tumor-specific deletion of TGF-β1 did not enhance anti-tumor immune responses (33). In contrast, others have reported that the deletion of T cell-derived TGF-β1 was sufficient to prevent tumor growth (32).

Although *Smad3* cKO mice did not mount immune responses as potent as those of DNTGFβRII Tg+ mice, B16-melanoma growth in *Smad3* cKO mice appeared comparable to that in *Pdcd-1* KO mice or anti-PD-1 antibody treated WT mice (Figure 7). While adoptive transfer of naïve antigen-specific T cell is known to confer minimal anti-tumor effects, transferred Smad3 cKO OT1 effectively controlled tumor growth (Figure 5a). Collectively, these results provide direct evidence that PD-1-mediated anti-tumor immunity

depends in part on *Smad3* activation and that *Smad3*-driven PD-1 up-regulation is relevant to tumor immune evasion. Our data clearly show that αPD-1 treatment decreases B16 tumor growth in WT mice (49,50) but not in *Smad3* cKO mice.

In sum, our data demonstrate a novel immunosuppressive function of TGF-β1 in regulating high-level PD-1 expression on T cells encountering cognate antigen. In addition to other suppressive roles for TGF-β1, TGF-β1 enriched in the tumor microenvironment may induce high levels of PD-1 on T cells as they encounter antigens on the tumor surface, reducing T cell effector function and limiting the anti-tumor T cell response. In addition, our data provide mechanistic understanding of the regulation of high-level PD-1 expression. While it is well known that T cells against intact antigen in the setting of chronic viral infections such as HCV and HIV or malignancy express very high levels of PD-1, it is not known how those high levels are induced. This study elucidates a mechanism through which the highest levels of PD-1 are induced. Indeed, high TGF-β1 serum levels are associated with worse disease outcome in HCV infection (66) and TGF-β1 expression is high in the tumor microenvironment of advanced stages of cancer, which may further limit the efficacy of T cells against disease in those settings (67-69). Given the potential for autoimmunity with PD-1 therapy, it is worth investigating whether inhibitors of Smad3 used in combination with other immunotherapeutic agents activate T cells expressing the highest levels of PD-1 rather than all T cells bearing PD-1. Since the PD-1^{hi} subset of TIL may in fact contain the highest proportion of true tumor-specific cells, these may be the most important target population for Smad3 blockade.

Methods

Mice

All animals were housed and handled in compliance with Johns Hopkins Animal Care and Use policy. C57BL/6 DNTGFβRII Tg+ and C57BL/6 *Cd4-Cre* transgenic mice were purchased from the Jackson Laboratory. CD45.1 congenic mice were purchased from National Cancer Institute (NCI) at Frederick. *Smad2* flox/flox (fl/fl) and *Smad3* fl/fl mice were generated by Se-Jin Lee's Laboratory at Johns Hopkins School of Medicine and backcrossed to C57BL/6 at least 6 generations. OT-I and OT-II mice were generous gifts from Drs. Charles Drake and Hyam Levitsky at Johns Hopkins School of Medicine. Age-matched female mice were utilized in all *in vivo* experiments.

Human and murine primary T cell Isolation and Culture

Human peripheral blood mononuclear Cells (PBMCs) were isolated from leukopheresis by Ficoll-Hypaque density gradient. Isolated human PBMCs were subjected for CD3+ T cell isolation by using the Pan T cell Isolation Kit (Miltenyi) as instructed in the manual. The isolated cells were activated for 72 hr with αCD3/αCD28 Dynabeads (Invitrogen) at a cell to bead ratio of 1:1 in RPMI+10% Fetal Bovine Serum (supplemented with HEPES buffer, Penicillin/Streptomycin, and L-glutamine). Murine CD4+ and CD8+ T cells were isolated from the spleen and lymph nodes using the Negative Selection Kit (Invitrogen), and were activated with plate-coated αCD3 (10μg/ml) and soluble αCD28 (2μg/ml) or with cognate Ova peptides in the presence irradiated splenocytes for 72 hr.

Transient Transfection and Luciferase Assay

Jurkat T cells (clone E6-1) were purchased from the ATCC and were kept as a frozen stock. 1.5×10^7 Jurkat T cells were transfected with 10 μg pGL-3 Firefly Luciferase Vector (Promega) and 1μg of pRL-TK Vector (Promega) by electroporation using Nucleofector II (Amaxa/Lonza). The cells were rested in a 6-well plate overnight and activated with plate-coated αCD3 (10 μg/ml) and soluble αCD28 (5 μg/ml) with or without rhTGF-β1 (50 ng/ml). After 24 hr, the cells were harvested and lysed followed by luminescence measurement using Dual-Luciferase Assay (Promega). Where indicated, the cells were co-transfected with empty vector (pSG-V5), TGF-βRI-His (Addgene plasmid #19161), TGF-βRII (Addgene plasmid #11766). For siRNA-mediated knock-down, the cells were co-transfected with 1.5 μM of siRNA for *Smad2* (Santa Cruz Biotechnology, SC-38374) and *Smad3* (Santa Cruz Biotechnology, SC-38376).

Cytokine and Drug Treatments

Human recombinant IL-1α, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, IL-21, IL-23, INF-α, IFN-γ, TGF-β1, and TNF-α were purchased from Peprotech and used at 5, 50, and 500 ng/mL. The data shown in Supplemental Figure 1a are using 500 ng/mL only because the relative effects of cytokines were not different at other doses. Primary human T Cells were treated with neutralizing TGF-β1 antibody (Abcam, 2Ar2) and with small molecule inhibitors, SB431542, Cyclosporin A (Sigma-Aldrich), and SIS3 (Calbiochem) for 1 hr prior to activation at the indicated concentration range.

Flow Cytometry

After indicated time of culture, human T cells were harvested and centrifuged at 400*g* (or 1500 rpm) for 5 minutes. The cells were washed in FACS buffer (1× PBS + 2% Fetal Bovine Serum) and stained with Aqua Viability Dye (Invitrogen) as instructed in the manual. After wash, the cells were stained with PD-1 PE (Biolegend), CD8 PerCP, CD4 Pacific Blue, CD3 FITC (eBioscience), and HLA-DR APC (eBioscience) or qDot605 (Invitrogen). The similar protocol was used for murine T cells and PD-1 PE, CD4 or CD8 PerCP, FITC (eBioscience), LAG3 APC or PacBlue, CD4 BV605, CD8 BV570, CD3 AF700, PD-1 PE Cy7, CD44 AF700 (Biolegend) were used for flowcytometery. For intracellular staining, the cells were treated with Foxp3/transcription factor staining buffet set (eBioscience) and stained with Foxp3 FITC, TNFα PE (eBioscience), IL-2 PE-CF594, or IFNγ APC (BD Biosciences).

Real-time qPCR assay

Total RNA was extracted from primary T cells under the indicated conditions using RNEasy Plus Kit (Qiagen). 100 ng of extracted RNA was reverse-transcribed using SuperScriptIII First-Strand Synthesis System (Invitrogen). Generated cDNA was subjected for real-time PCR assay. *Pdcd-1* primer sequences are Forward 5′- CACTGAGGCCTGAGGATGG-3′; Reverse 5′-AGGGTCTGCAGAACACTGGT-3′. All target genes were normalized to 18s rRNA or 28s rRNA as previously described.

Molecular Cloning and Site-directed Mutagenesis

Human PD-1promoter (1.9 kbases) was cloned from genomic DNA of isolated CD3+ T cells and the sequence was confirmed. The amplified clones were ligated to SacI/XhoI digested pGL3-Basic Vector (Promega) using In-Fusion Cloning Kit (Clonetech). Site-directed mutagenesis was carried out using following primers for NFAT, SBE-D, SBE-P sites using QuickChange Lightning Kit (Agilent Technologies). Forward: 5′-GATGCTCTTTTTGGACTGTTTCGG-3′ Reverse 5′-CCGAAACAGTCCAAAAAGAGCATC-3′ (NFAT); Forward: 5′-ACCTTAGCTGGATGGCAGCA-3′ Reverse 5′-TGCTGCCATCCAGCTAAGGT-3′ (SBE-D), Forward: 5′-CGCGCCTCGCATCCATCATCTT-3′ Reverse: 5′-AAGATGATGGATGCGAGGCGCG-3′ (SBE-P).

Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was performed according to the manufacturer's guidance (Invitrogen MAGnify ChIP system). Briefly, isolated CD3+ T cells were activated with αCD3/αCD28-conjugated beads for 24 hr and fixed with 2% formaldehyde. Sonicated DNA was immunoprecipiated with α*Smad3* (Cell Signaling Technology), and αNFATc1 (Santa Cruz Biotechnology). The immunoprecipated chromatin was analyzed on Roche Light Cycler 480 by SYBR green using the following primers for PD-1 promoter. *Pdcd-1*: Forward 5′-CCTCACATCTCTGAGACCCG-3′; Reverse 5′-CCGAAGCGAGGCTAGAAACC-3′ *Gapdh*: 5′-TACTAGCGGTTTTACGGGCG-3′ 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′

Western Immunoblotting

Human or murine T cells were activated as indicated and harvested and lysed in RIPA Buffer (Cell Signaling Technology). Protein-extract concentrations were measured using BCA protein assay kit (Thermo Scientific) and followed by heating under reducing conditions. The equal amounts of extracts were loaded/run on NuPAGE Precast gels (Invitrogen) and transferred membranes were blotted with following antibodies: p-*Smad2*, total *Smad2*, total *Smad3* (Cell Signaling Technology), and β-actin (Sigma).

B16 melanoma and adoptive T cell transfer experiments

B16 melanoma cell lines were purchased from the ATCC and kept as a frozen stock. 1×10^5 B16 melanoma cell lines were injected on a flank in 100 μl volume. Tumor volumes were measured every other day using a caliper and assessed using the formula ½ (Length×Width^2). CD45.1 host mice were injected with 1×10^5 B16-Ova melanoma cell lines on a flank. 8×10^6 WT OT-1 or cKO OT-1 were labeled with CellTrace CFSE Cell Proliferation Kit (Life Technologies) and were adoptively-transferred in to tumor-bearing mice by retroorbital injection on Day 12. The tumors were harvested on Day 5 after the adoptive transfer and lymphocytes were purified using Percoll (GE Healthcare) gradient. In a blocking experiment, 5×10^5 B16 melanoma cells were injected on a flank and 100 μg Armenian hamster IgG isotype control (Rockland) or anti-PD1 antibody (G4) were injected intraperitoneally two times a week from Day 0.

MC38 colon adenocarcinoma experiments

MC38 colon adenocarcinoma cell lines were purchased from the ATCC and kept as a frozen stock. 4×10^5 B16 melanoma cell lines were injected on a flank in 100 μl volume. Tumor volumes were measured using a caliper and assessed using the formula ½ (Length×Width^2). The tumors were harvested on Day 23 and lymphocytes were purified using Percoll (GE Healthcare) gradient. For intracellular cytokine staining, lymphocytes were stimulated with phorbol 12- myristate 13- acetate (PMA, 50 ng/ml) and inomycin (500 ng/ml) in the presence of Brefeldin A and Monensin (eBiosciences) for 4 hours prior. After stimulation, cells permeablizied and stained for intracellular cytokines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Engagement of the co-inhibitory receptor PD-1 of its ligand, PD-L1, dramatically inhibits the anti-tumor function of TIL within the TME. Our findings represent a novel immunosuppressive function of TGF-β and demonstrate that TGF-β1 allows tumors to evade host immune responses in part through enhanced *Smad3*-mediated PD-1 expression on TIL.

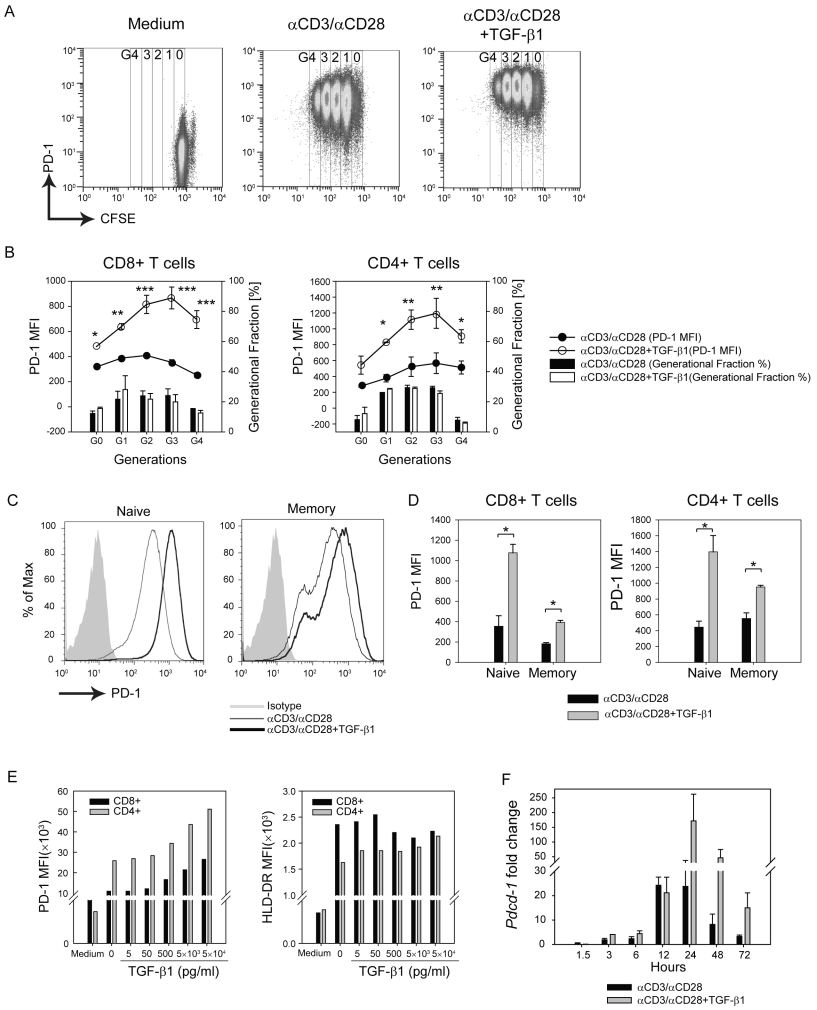

Figure 1.

TGF-β1 enhances PD-1 expression on human T cells in a dose-dependent manner. Human CD3+ T cells were isolated from healthy donor PBMCs and were activated with αCD3/αCD28-conjugated beads with or without TGF-β1 (50 ng/ml). (a) Representative plots PD-1 (Y-axis) vs. CFSE (X-axis) are shown for different conditions. (b) PD-1 MFI is shown as filled circle lines (αCD3/αCD28) and open-circle lines (αCD3/αCD28 + TGF-β1). The percentage of cells in each CFSE generation (G0, G1, G2, G3 and G4) is shown as black bar graphs (αCD3/αCD28) and white bar graphs (αCD3/αCD28 + TGF-β1). The data represent combined results of two independent trials. (c) Naïve and memory subset of T cells (CD4+ and CD8+ T cells) were isolated based on CCR7 and CD45RA expression and treated with αCD3/αCD28 activation in the presence or absence of TGF-β1. The representative histogram of PD-1 is shown: shaded histogram (Isotype); thin histogram (αCD3/αCD28); and bold histogram (αCD3/αCD28+TGF-β1). (d) PD-1 MFI was assessed on each subset (X-axis) of CD8+ (left) and CD4+ (right) T cells; αCD3/αCD28 alone (black bars); αCD3/αCD28 with TGF-β1 (grey bars). The data represent combined results of two independent trials. (e) Isolated human CD3+ T cells were activated with αCD3/αCD28-conjugated beads in the presence of varying concentrations of TGF-β1 (5 to 5 × 10^4 pg/ml).

Mean fluorescent intensity (MFI) of PD-1 (left) and HLA-DR (right) expressions were assessed in CD4+ (grey bars) and CD8+ (black bars) T cells. The shown result is the representative of at least three independent trials. (f) *Pdcd-1* transcript levels of human CD3+ T cells under different treatments were normalized to that of resting CD3+ T cells. The result is shown as mean +/− SEM of technical replicates and representative of at least three independent trials. The data were analyzed using Student's t-test and considered significant if *P<0.05, **P<0.01, ***P<0.001.

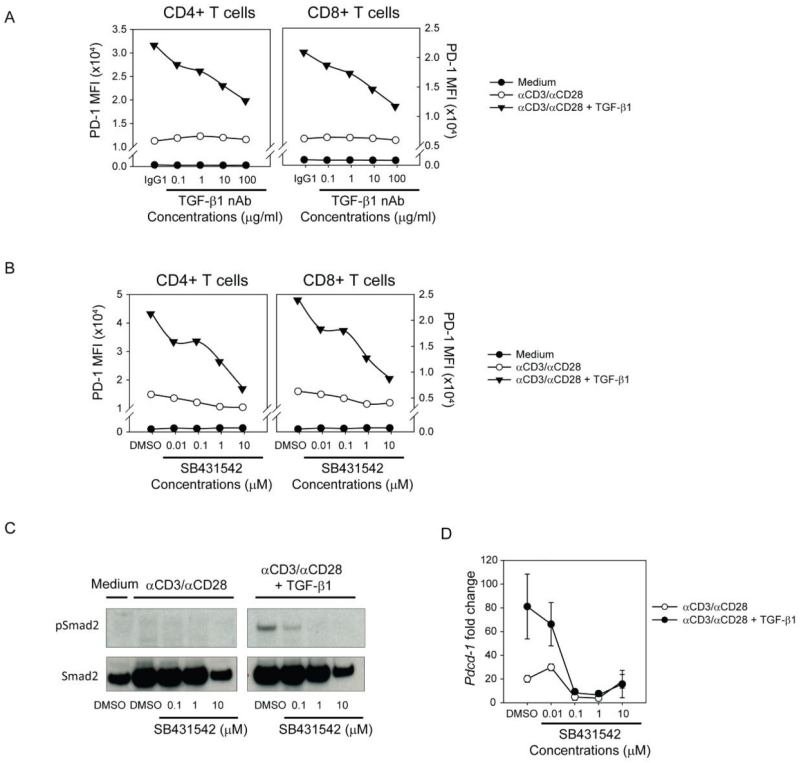

Figure 2.

Anti-TGF-β1 neutralizing antibody and a TGF-βRI kinase inhibitor negate TGF-β1 mediated PD-1 enhancement

(a, b) Enriched human CD3+ T cells were activated with αCD3/αCD28-conjugated beads and TGF-β1 under varying concentrations of TGF-β1 nAb (a) or SB431542 (b) and PD-1 MFI was assessed: medium alone (closed circles) αCD3/αCD28 only (open circles); αCD3/αCD28 + TGF-β1 (closed triangles). The result shown is representative of at least three independent trials. (c) Western-blot analysis of phosphorylated-*Smad2* (p*Smad2*) in human CD3+ T cells treated with varying concentrations of TGF-βRI kinase inhibitor (SB431542). (d) Enriched human CD3+ T cells were activated with αCD3/αCD28-conjugated beads and TGF-β1 under increasing concentrations SB431542. *Pdcd-1* transcript levels in each condition were normalized to that of resting human CD3+ T cells. The result is shown as mean +/− SEM of technical replicates and representative of at least three independent trials.

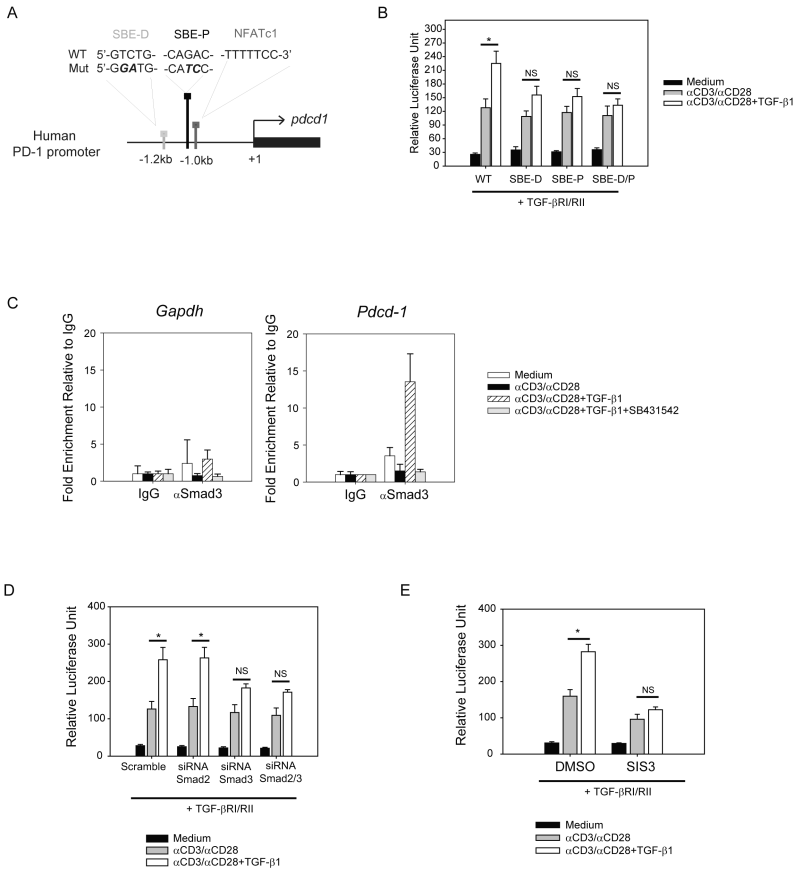

Figure 3.

Smad3 directly binds to the Smad-binding elements (SBEs) and regulates PD-1 promoter activity

(a) Schematic illustration of the proximal region of human *Pdcd-1* promoter. Two Smad-binding-elements (SBEs) are located at 1.2 kb (SBE-D) and 1.0 kb (SBE-P) upstream of the *Pdcd-1* transcription start site. NFATc1 consensus sequence is located in immediate proximity to SBE-P. Wild-type (WT) and mutated (Mut) sequences of both SBE-D and SBE-P are shown. (b) Jurkat T cells were transfected with luciferase reporter vectors containing wild-type (WT), mutant SBE-D, mutant SBE-P, and mutant SBE-D/P sequences of PD-1 promoter (1.9 kb). After co-transfection with TGF-βRI and RII expression plasmids, the cells were activated with plate-bound αCD3 and soluble αCD28 in the absence (grey bars) or presence (white bars) of TGF-β1 (50 ng/ml). Luciferase activity was measured as described in the method. (c) Isolated human CD3+ T cells were activated under different conditions: medium alone (white bars); αCD3/αCD28 alone (black bars); αCD3/αCD28 with TGF-β1 (hatched bars); αCD3/αCD28 + TGF-β1 with SB431542 (grey bars). Immunoprecipitated DNA was subjected for qPCR and fold enrichment of binding relative to IgG is shown as mean +/− SEM of triplicate results. (d) Jurkat T cells were co-transfected with 1.5 μM of siRNA against *Smad2* or *Smad3* and PD-1 promoter-driven luciferase activity was measured in relative luciferase units (ratio of firefly to renilla luciferase activity). (e) Transfected Jurkat T cells were treated with 10μM of specific inhibitor of

Smad3 (SIS3) and PD-1 promoter-driven luciferase activity was measured after activation. The results are shown as mean +/− SEM of technical replicates and is representative of at least three independent trials. The data were analyzed using two-way analysis of variance (ANOVA) and considered significant if *$p<0.05$.

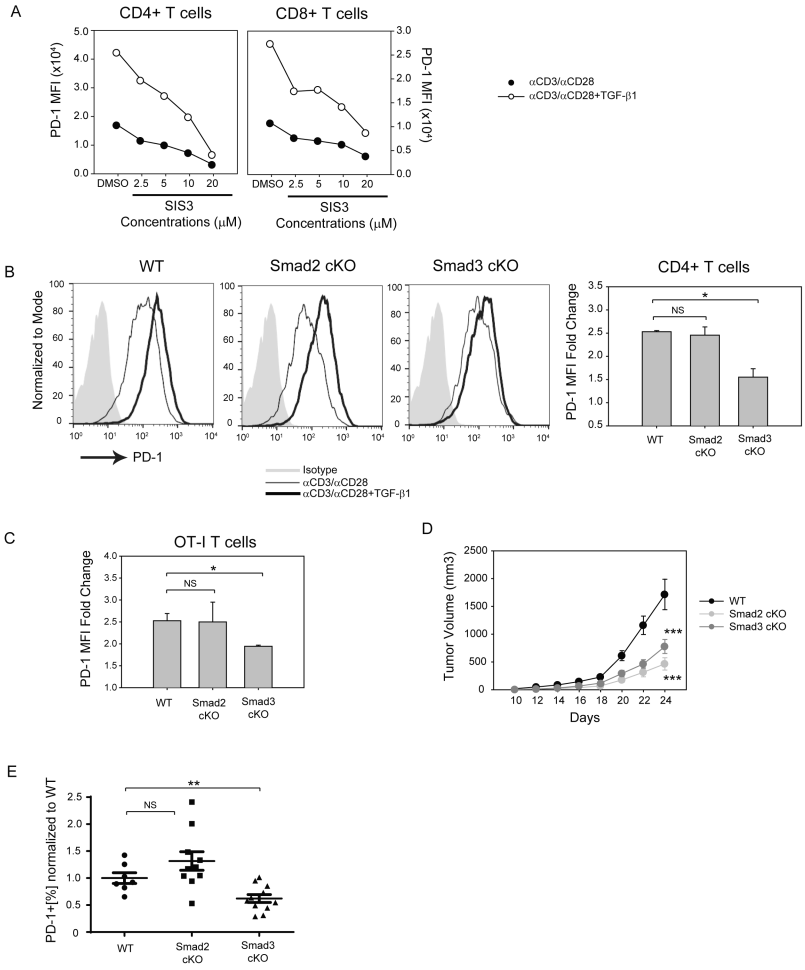

Figure 4.

TGF-β1-dependent *Smad3* enhances PD-1 expression on human and murine T cells

(a) Human CD3+ T cells from healthy donors were isolated and pretreated with SIS3 at varying concentrations. Subsequently, the cells were activated with αCD3/αCD28-conjugated beads with or without TGF-β1. Mean fluorescence intensity (MFI) of PD-1 expression in different conditions was assessed: αCD3/αCD28 (closed circles); αCD3/αCD28 + TGF-β1 (open circles). The result shown is representative of at least three independent trials. (b) CD4+ T cells were isolated from wild-type (WT), *Smad2* f/f; *Cd4*-cre (*Smad2* cKO), *Smad3* f/f; *Cd4*-cre (*Smad3* cKO) mice and activated with plated-coated αCD3 and soluble αCD28 with or without TGF-β1 (50 ng/ml). PD-1 expression is shown as overlaid histograms with shaded histogram (isotype control), thin histogram (αCD3/αCD28), bold histogram (αCD3/αCD28+TGF-β1). PD-1 MFI is also shown as mean +/− SEM and represents combined results of two independent trials (bar graphs). (c) Isolated WT, *Smad2* cKO, and *Smad3* cKO OT-1 cells were activated with type-1 ovalbumin in the presence of irradiated splenocytes. PD-1 MFI is shown as mean +/− SEM and represents combined results of two independent trials (bar graphs). (d) Growth kinetics of B16 melanoma in WT (n=7), *Smad2* cKO (n=10), and *Smad3* cKO (n=11) mice are shown as the mean volume +/− SEM on different days. The data represent the combined results of two

independent experiments. (e) Average CD8+ PD-1+ percentages in *Smad2* cKO and *Smad3* cKO TIL are shown as normalized values to WT CD8+ PD-1+ percentages. The data were analyzed using Student's t-test and considered significant if *p<0.05, **p<0.01, ***p<0.001.

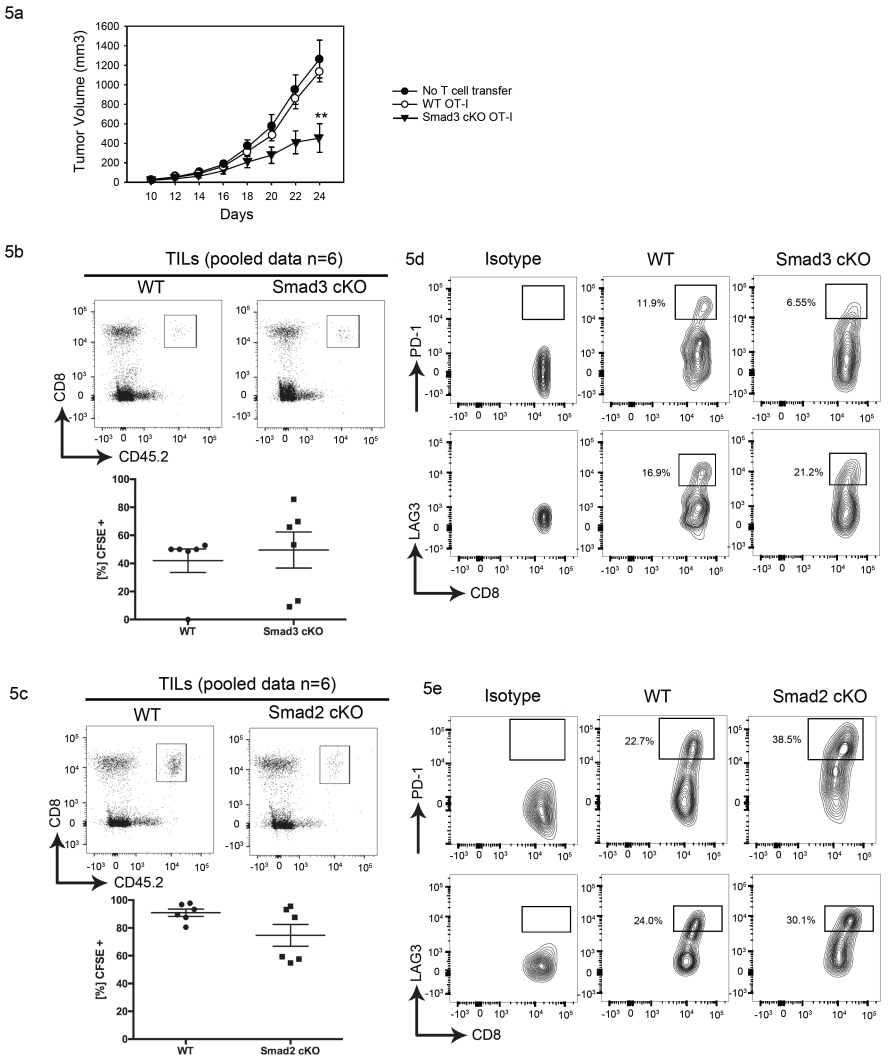

Figure 5.

Adoptive transfer of *Smad3* cKO CD8+ T cells results in reduced tumor burden and PD1^{hi} subset relative to transfer of WT CD8+ T cells

(a) Growth kinetics of B16-Ova in CD45.1 congenic mice that received no T cells (closed circles), WT OT-1 (open circles) or *Smad3* cKO OT-1 (triangles). C57/BL6 expressing CD45.1 congenic markers were challenged with 1×10^5 B16-Ova melanoma cell line on Day 0. On Day 10, 10^7 CD45.2 CD8+ OT-1 T cells from WT (n = 7) or *Smad3* cKO (n = 5) mice were adoptively transferred into the mice with comparable tumor sizes. Tumor volume (mm^3) is shown as mean +/− SEM on different days, and the data represent combined results of two independent experiments. The data were analyzed using one-way ANOVA and considered significant if *p<0.05, **p<0.01. (b, c) CFSE-labelled tumor infiltrating WT OT-1 (top) or cKO OT-1 (bottom) were isolated from B16-Ova 5 days after adoptive transfer, and tumor infiltrating lymphocytes (TIL) proliferation was assessed: *Smad3* cKO (b) and *Smad2* cKO OT-1 (c). CD45.2+ donor population was gated from a plot of CD8 (Y-axis) and CD45.2 (X-axis) (left), and a representative histogram of CFSE (right) is shown from pooled TIL from n = 6 mice per group. (d, e) Contour plots of PD-1 (top) and LAG3 (bottom)

among the proliferated cells (i.e CFSE negative populations) are shown as isotype (left), WT (middle) and cKO (right): *Smad3* cKO (d) and *Smad2* cKO (e). The data are representative of two independent experiments.

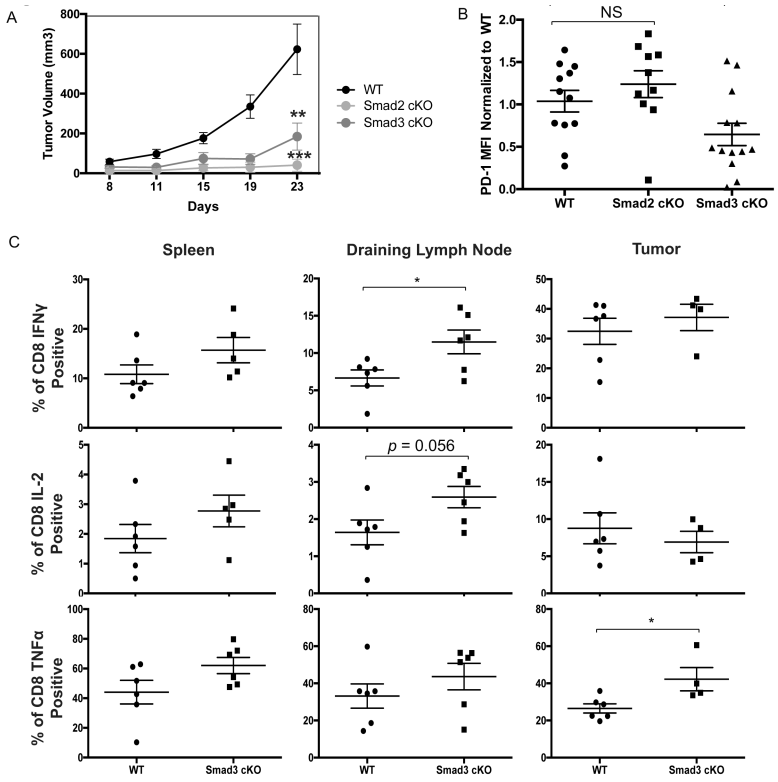

Figure 6.

Loss of *Smad3* in CD8+ T cells leads to enhanced cytokine production.

(a) Growth kinetics of MC-38 in WT (n=7), Smad2 cKO (n=6), or Smad3 cKO cells (n=6) are shown as the mean volume +/− SEM on different days. Data is representative example from 2 independent experiments. (b) Average CD8+ CD44+ PD-1 MFI in *Smad2* cKO and *Smad3* cKO TIL are shown as normalized values to WT CD8+ PD-1+ percentages. The data were analyzed using Student's t-test and considered significant if *p<0.05. (c) Percentage of CD8 T cells producing IFNγ, TNFα, or IL-2 in the spleen, draining lymph node, and tumor. The data were analyzed using Student's t-test and considered significant if *p<0.05.

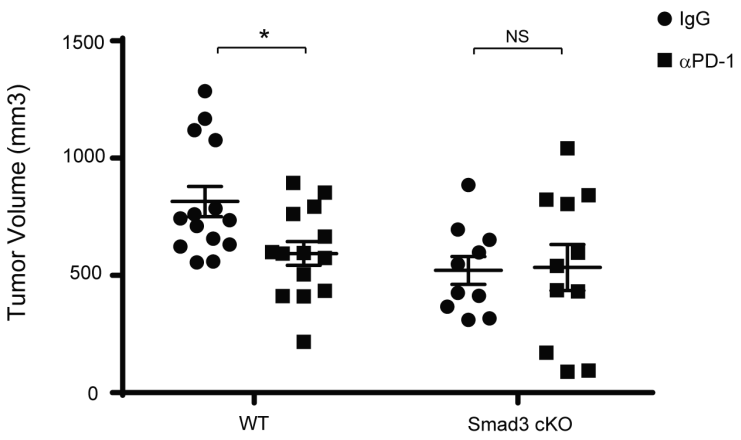

Figure 7.

Anti-PD-1 blocking antibody enhances anti-tumor immune responses in WT mice, but has minimal effect in *Smad3*-deficient mice.

WT and *Smad3* cKO were challenged with B16-melanoma cell line on Day 0. WT and *Smad3* cKO mice were treated with either isotype-matched control IgG (circles) or anti-PD-1 antibody (squares) from the day of tumor implantation until Day 17. Tumor volume (mm^3) on Day 17 is shown as mean +/− SEM on and the data represent combined results of two independent experiments. The data were analyzed using two-way analysis of variance (ANOVA) and considered significant if *$p<0.05$.